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EFFECT OF UV-C ON TWO CELL LINES FROM SUGARBEET

BY

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ABSTRACT

Effect of UV-C on two cell lines from sugarbeet.- Two different sugarbeet calli were exposed to UV-C for short times. The first one, a hormone-dependent, non-organogenic callus (N), produced significant amounts of hydrogen peroxide even after short irradiations. The second one, a habituated, hormone-independent non-organogenic callus (H), appeared to be much less reactive and produced only weak amounts of H_2O_2 after rather long exposures to UV-C. It was possible to isolate a substance as yet unidentified, which was less abundant in N than in H cells and which disappeared partly after UV-C treatments. Peroxidases from different cell compartments were measured 24 hours after UV-C irradiations, using guaiacol, ferulic acid or ascorbic acid as electron donors. In most cases, UV-C produced a decrease of activity. The most important effect was observed on ascorbate peroxidase activity which decreased strongly in every compartments of both cell types. This effect of UV-C could be explained by the presence of increased amount of H_2O_2 in the apoplast.

Key-words: Tissue culture, Habituation, Hydrogen peroxide, Chemiluminescence, Peroxidases, Bilins.

INTRODUCTION

Exposure of plants or cultured plant cells to ultraviolet radiation (UV) of various wavelengths brings about many physiological effects (CALDWELL, 1971; ROZEMA *et al.*, 1997). These effects include changes in ion permeability (MURPHY & WILSON, 1982; KARCZ & STOLAREK, 1988) or gene transcription (STRID, 1993), and inhibition of photosynthesis (BORNMAN, 1989; CALDWELL, 1971). The production of activated oxygen species is also a consequence of an irradiation with UV. It may be observed in animals (BERTLING *et al.*, 1996) and in plants (MURPHY & HUERTA, 1990) in which it induces an oxidative stress (PENEL, 1997). The accumulation of hydrogen peroxide has been reported to occur in whole plants, but also in suspension cultures of cells, following UV irradiation (MURPHY & HUERTA, 1990; RAO *et al.*, 1996). The question arises to know whether each type of cells respond to UV irradiation by such a production of H_2O_2, or if there are differences according to the physiological state or the redox status of the cells. To address this problem, we have submitted two different

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cell lines from sugarbeet to short irradiations with UV-C. One is a normal non-organogenic callus (N), the other one is a fully habituated, non-organogenic callus (H) growing without the supply of exogenous hormones. Both were established from leaf pieces of sugarbeet almost twenty years ago (DE GREEF & JACOBS, 1979) and kept their main properties since that time. Although they have been derived from the same plant and exhibit similar protein composition (TACCHINI *et al.*, 1995), these two calli are quite different in their ultrastructure (CREVECOEUR *et al.*, 1992), metabolisms and physiology (LE DILY *et al.*, 1993; GASPAR *et al.*, 1988). N callus is hormone-dependent for its growth and has a rather common behaviour. H callus, in addition to its complete independence with respect to exogenously supplied hormones, exhibits many of the characteristics of a cancerous state (GASPAR *et al.*, 1991). One of the most striking differences between these two calli concerns their redox properties. Whereas N cells produce much apoplastic H_2O_2 and reduce only weakly extracellular ferricyanide, N cells synthesize little H_2O_2 and exhibit a strong reducing capacity (CARRIÉ *et al.*,1994). It appeared interesting to study the response of these two cell lines to an exposure to UV radiation, since their redox status is quite different. An explanation of the difference of response observed between N and H cells was tentitatively looked for in their different tetrapyrrole metabolism (HAGEGE *et al.*, 1992).

MATERIAL AND METHODS

All the experiments were performed on cells from sugarbeet (*Beta vulgaris* L., *altissima*) N and H calli. The experimental conditions to obtain and subculture these calli were already described in detail (KEVERS *et al.*, 1981). Forteen-day old calli were used for the experiments.

Ultraviolet irradiations (UV-C) were performed with a 15 W low pressure tube (50 $\mu W/cm^2$ at 254 nm). Petri dishes containing the calli were opened aseptically during UV exposure and then closed until cells were used. Stock solutions of biliverdin and bilirubin (Sigma) were prepared in methanol and diluted in water before addition on cells, which were then irradiated.

Apoplastic hydrogen peroxide was quantified by chemiluminescence in the presence of luminol, as described previously (CARRIE *et al.*, 1994), using 500 mg of cells dispersed in 1 ml Tris buffer at pH 8.6. Peroxidase activities were determined in three different fractions prepared as follows. One gram of callus was dispersed in 1 ml of 20 mM Hepes buffer pH 7. After a few min of incubation, the liquid which contained the soluble extracellular peroxidases was separated by centrifugation at 1'000 g for 5 min (fraction 1). Then the cells were ground in Hepes buffer pH 7, containing 1 M NaCl. After a centrifugation at 8'000 g for 10 min, the supernatant was used as fraction 2, containing mainly the ionically bound proteins. The pellet was then resuspended in a buffer containing 2% cellulase and 1% macerozyme (R10, Yakult Pharmaceutical, Japan). After an incubation overnight, the preparation was centrifuged at 8'000 g for 10

min. The supernatant contained the covalently bound proteins (fraction 3). Peroxidase activity was assayed in the three fractions using 8 mM guaiacol (2-methoxyphenol) and 2 mM H_2O_2 in 50 mM phosphate buffer pH 6.1 (ΔA470/min.mg protein) (KIEFER *et al.*, 1985), or 2 mM ferulic acid (4-hydroxy-3-methoxycinnamic acid) and 5 mM H_2O_2 in acetate buffer pH 4.5 (ΔA310/min.mg protein) (TAKAHAMA, 1995), or 0.4 mM ascorbic acid and 2 mM H_2O_2 in phosphate buffer pH 6.1 (ΔA265/min.mg protein) (CASTILLO *et al.*, 1986).

Samples of N and H calli were ground in methanol (1 gr/1 ml LiChrosolv, Merck). The extracts were centrifuged at 1'000 rpm for 5 min. Methanol was evaporated under nitrogen. The resulting dry residue was dissolved in 50 µl methanol. After centrifugation at 1'000 rpm for 3 min, the sample was ready for HPLC separation. Five µl were injected into a HPLC column (Res Elut-BD5 C18, Varian). Methanol was used as eluent (flow rate 0.1 ml/min).

RESULTS AND DISCUSSION

Samples of N and H calli were exposed to UV-C during various times to determine their sensitivity to this treatment (Fig. 1). When measured one hour after the irra-

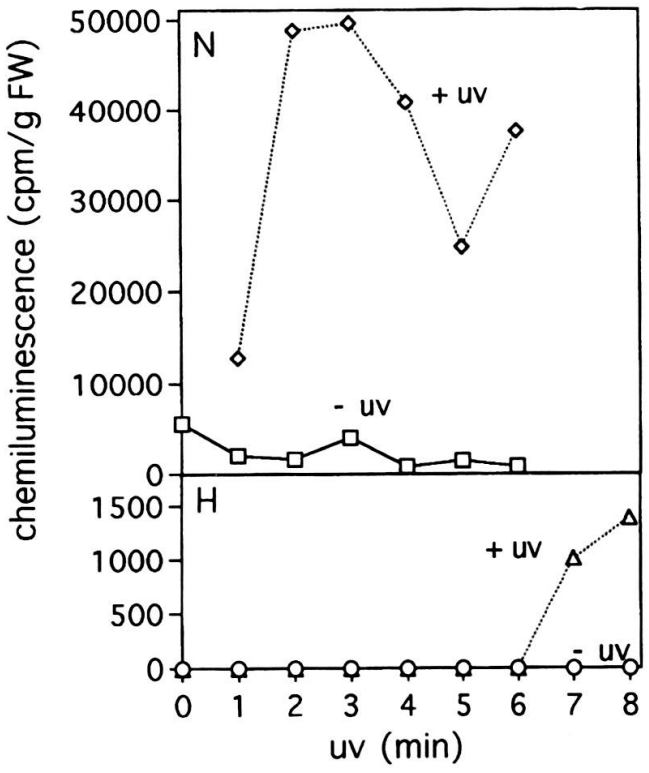

FIG. 1.

Effect of different durations of exposure to UV-C on the chemiluminescence level of N and H calli, measured 60 min after the treatment.

diations, the chemiluminescence of N callus was maximum after an exposure of 2-3 minutes and somewhat less important following longer treatments. H callus exhibited a small response only after 7-8 minutes. In the conditions used in the present work, the basal level of H callus chemiluminescence was near zero. Values for N cells in the absence of UV were comprised between 800 and 6000 cpm. These data are in accordance with those previously published (CARRIE *et al.*, 1994). The addition of catalase (10 units/ml) reduced the chemiluminescence by 65 per cent (data not shown), confirming that it is mainly due to the presence of H_2O_2.

The level of chemiluminescence produced by sugarbeet cells in the presence of luminol was measured after exposure to UV-C (Fig. 2). After a strong incresase during the first hour following UV, N cells exhibited a fluctuating response. H callus showed a very slow and regular increase of chemiluminescence, which remained at a low level. In the days after a single UV-C treatment, N cells did not exhibit macroscopic signs of injury, whereas H callus presented many necroses. The data shown in Figs. 1 and 2 indicated that UV-C applied to plant cells for a short time induced the formation of H_2O_2, as already reported (MURPHY & HUERTA, 1990). The data indicated also that the extent of the response differed according to the type of cells which was irradiated. The resistance was also dependent on the cell type. Surprisingly, N cells, which accumulated much more H_2O_2 than N cells, were less injured, suggesting that the level of this reduced oxygen species is not the only factor explaining the appearance of necrotic lesions.

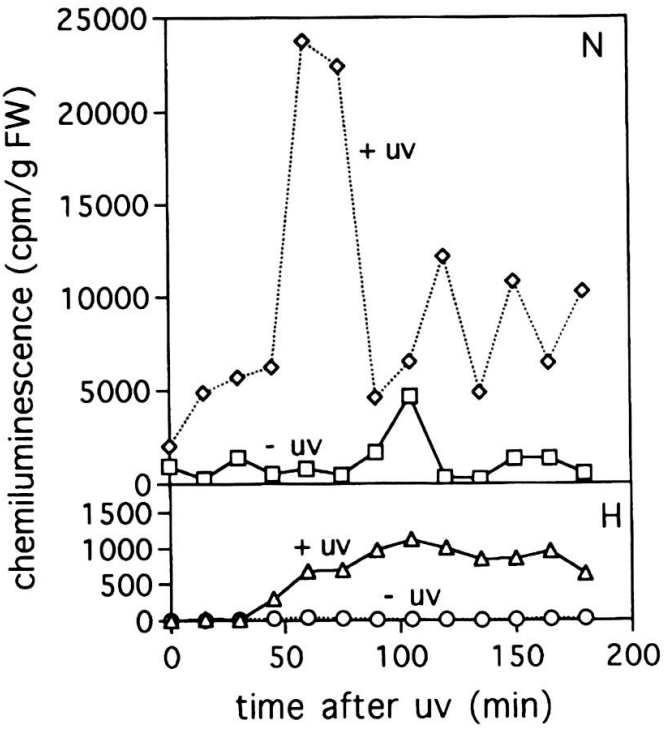

FIG. 2.
Levels of chemiluminescence measured at different times after an exposure to UV-C of 2 min (N callus) or 8 min (H callus).

It is known that UV irradiation induces the formation of activated oxygen species in mammalian cells, and that bilirubin or biliverdin are produced to protect cells against oxidative stress (KEYSE & TYRRELL, 1989). Phycobiliproteins of cyanobacteria were also affected by an exposure to UV radiations (SINHA *et al.*, 1995). On the other hand, it has been reported that N and H calli differ by their tetrapyrrole metabolism (HAGEGE *et al.*, 1992). H cells exhibit a general deficiency in hemoproteins and chlorophylls. This particularity may be explained by the abnormal structure of their chloroplasts (CREVECOEUR *et al.*, 1992). Both types of cells use the Shemin pathway for their tetrapyrrole biosynthesis, which is rather unusual for plant cells (BISBIS *et al.*, 1997). The possibility exists also that this disturbed porphyrin metabolism results in the accumulation of bilin type compounds, which could play a role in the response of H cells to UV irradiations. In order to test this hypothesis, N cells were exposed to UV-C in the presence of bilirubin and biliverdin (Fig. 3), two products of heme degradation which are known to play a protective role in animal cells. These two molecules reduced

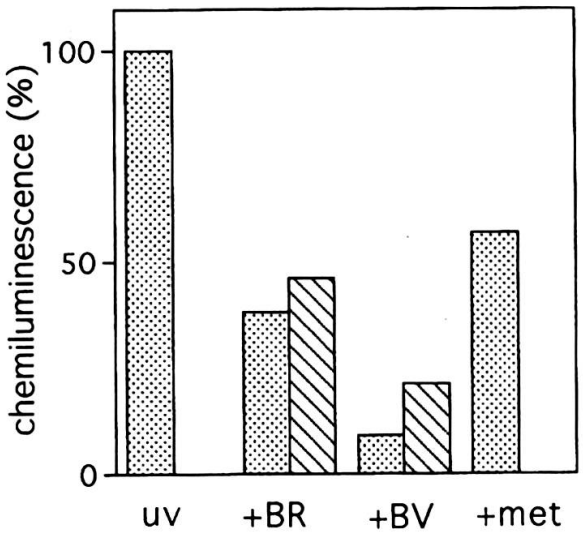

FIG. 3.

Effect of bilirubin (BR, 50 and 5 µM), biliverdin (BV, 50 and 5 µM) and methanol (met, 1%) on the level of chemiluminescence of N callus exposed to 2 min UV-C. Measurements were performed 60 min after irradiations.

significatively the extent of the chemiluminescence response, suggesting that they decreased the formation of H_2O_2 upon exposure to UV-C, maybe by scavenging the newly formed oxygen species. Methanol, used to solubilize these two bilins had also an effect. These observations were in accordance with the known antioxidant properties of bilins (STOCKER, 1987).

Attempts to detect either biliverdin or bilirubin in sugarbeet calli, using commercial detection kits, or to find a heme oxygenase activity remained unsuccessful. The research of similar compounds was undertaken. Substances extracted with methanol from N and H cells were separated by HPLC, using a detection at 327 nm. In the

conditions used a major peak was detected during the elution with a retention time of 6.3-6.4 minutes. Biliverdin in the same conditions came out at about 6 minutes. The substance separated by this procedure was quantified in N and H calli at various times after exposure to UV-C (Fig. 4). H cells contained a significatively higher amount of this unknown substance. UV-C reduced rapidly its amount, down to a level similar to

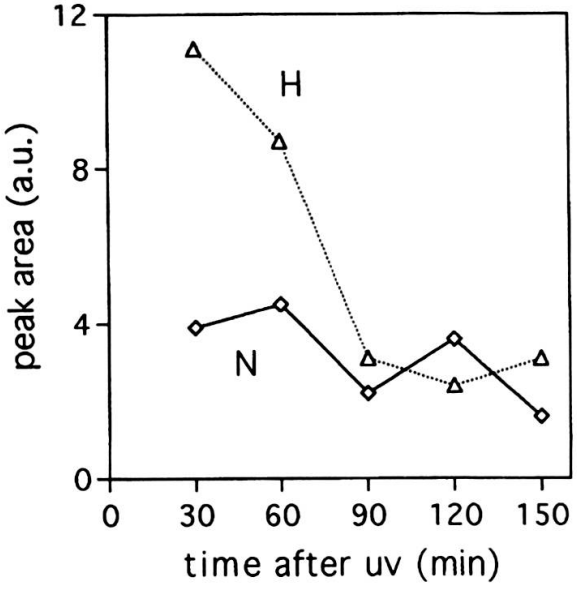

FIG. 4.

Effect of an exposure of N and H calli to UV-C on the amount of the unknown substance extracted from one gram of callus at various times after the treatment. The substance separated by HPLC had a retention time of 6.3-6.4 min, and was detected at 327 nm. Peak area was expressed in arbitrary units.

the level of N cells within 90 minutes. The rather low amount of substance present in N cells was not much affected by UV-C. This substance has not been identified. It presented two absorption maxima at 276 and 327 nm (Fig. 5).

Apoplastic H_2O_2 formed in response to a short UV-C irradiation is the substrate of peroxidases, enzymes which are known to be mainly present in cell wall and extracellular spaces (GASPAR *et al.*, 1982). An analysis of peroxidase activity was therefore performed in order to see if there was an effect of UV-C on these enzymes. As peroxidases are known to be able to use various electron donors to reduce H_2O_2, guaiacol, ferulic acid and ascorbic acid were tested with enzymes prepared from extracellular spaces and cells (Fig. 6). The general effect of UV-C on peroxidases was a decrease of activity in almost each case. This was particularly obvious when ascorbic acid was used as electron donor. Twenty four hours after an exposure to 2 min UV-C, the ascorbate peroxidase activity was decreased by 80 to 88 per cent in all fractions except H3. When determined with guaiacol or ferulic acid, the peroxidase activity exhibited also a decrease in UV-C irradiated N cells. The effect of the treatment was less important on these peroxidase activities in H cells. This difference between the two types of calli

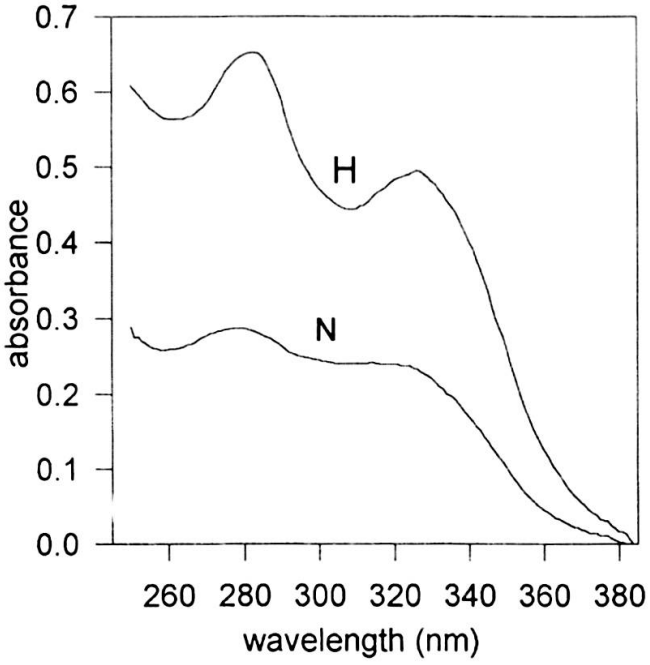

FIG. 5.

Absorption spectra of the unknown substance separated by HPLC from 5 gr of N and H calli.

could be explained by the level of H_2O_2 produced in reaction to UV-C. A one- or two-minute irradiation induced an important formation of H_2O_2 in N callus apoplast, but not in H callus (Fig. 1). This would be consistent with the report showing that an increase of H_2O_2 concentration in apoplast induced a degradation of proteins and an inactivation of many enzymes (GOMEZ *et al.*, 1995). The most striking differences were observed when ascorbic acid was used as electron donor. This particular peroxidase activity is normally due to ascorbate peroxidases (E,C, 1.11.1.11) which are distinct from the secreted plant peroxidases (E.C. 1.11.1.7), but the latter are also able to oxidise ascorbate in certain conditions (MELHORN *et al.*, 1996). The present results do not allow to know which kind of enzymes was measured, but it appeared clearly that this activity was the most sensitive to an exposure to UV-C, which could act directly on the protein or indirectly through the formation of H_2O_2. The main or unique function of ascorbate peroxidase is H_2O_2 scavenging (PENEL, 1997). In addition, the results presented in Fig. 6 provided confirmation that peroxidase activity is greater in N than in H callus (KEVERS *et al.*, 1981).

In conclusion, the level of formation of H_2O_2 in cells exposed to UV-C appeared to be different in different cells. This variability could result from the presence of an unknown substance which would have a protective effect. Further work will be necessary to identify the chemical nature of this product.

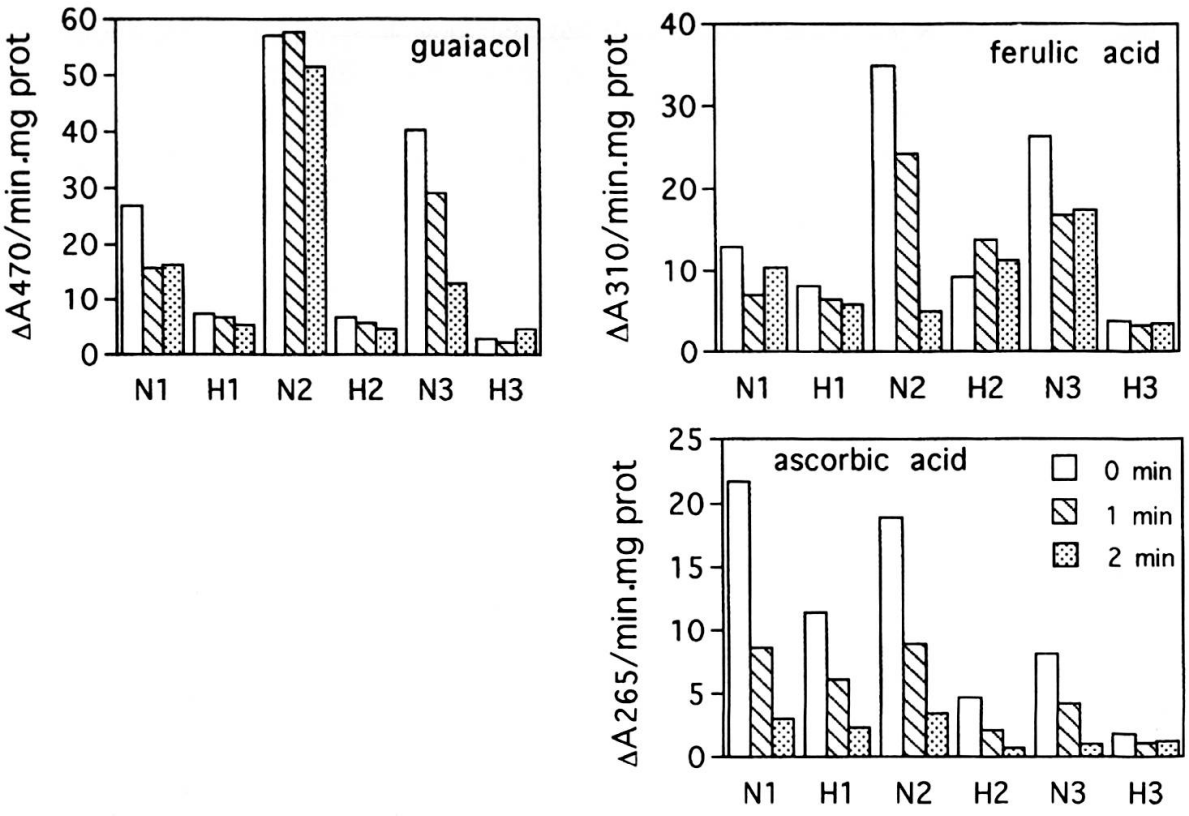

FIG. 6.

Peroxidase activities in fractions 1, 2 and 3 (see material and methods) measured with guaiacol, ferulic acid and ascorbic acid as electron donors. Control calli (0 min) and calli irradiated during 1 or 2 min were used 24 hours after UV-C.

RÉSUMÉ

EFFET DE L'UV-C SUR DEUX LIGNÉES CELLULAIRES DE BETTERAVE

Deux cals de betterave différents ont été exposés à un rayonnement UV-C pendant des temps courts. L'un deux, non-organogène croissant seulement en présence d'un apport d'hormones (N), produit des quantités importantes de peroxyde d'hydrogène en réponse à de courtes expositions aux UV-C. L'autre, cal habitué également non-organogène dont la croissance est indépendante d'hormones exogènes (H), ne produit que de faibles quantités de H_2O_2 en réponse à une exposition relativement plus longue à UV-C. Une substance inconnue, dont la quantité diminue très nettement dans le cal H après exposition à UV-C, a été isolée dans les deux types de cals. L'activité peroxydasique extraite de différents compartiments sub-cellulaires a été mesurée 24 heures après une expostion à UV-C en utilisant le gaïacol, l'acide férulique et l'acide ascorbique comme donneurs d'électrons. Il ressort des mesures que l'UV-C a provoqué une diminution d'activité dans presque tous les cas. L'effet le plus important a été observé

sur l'activité ascorbate peroxydase, qui décroît dans les divers compartiments des deux types de cellules. Cet effet de l'UV-C pourrait être dû à l'augmentation de H_2O_2 dans l'apoplaste.

Mots-clés: Culture de tissu, habituation, peroxyde d'hydrogène, chemiluminescence, peroxydases, bilines.

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